ECOLOGY AND THERMAL INACTIVATION OF MICROBES IN AND ON INTERPLANETARY SPACE VEHICLE COMPONENTS

Forty-first Quarterly Report of Progress

Order No. W-13411

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INTRODUCTION

The following manuscript has been submitted to the journal, Applied Microbiology, for publication. It represents a summary of the work done under this contract on the inactivation of Bacillus subtilis var. niger under conditions of high humidity.

CHANGES OCCURRING DURING THERMAL INACTIVATION OF *BACILLUS SUBTILIS* VAR. *NIGER* SPORES IN AQUEOUS SUSPENSION

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ABSTRACT

Spores of *Bacillus subtilis* var. *niger* were heat treated in aqueous suspension at 90 C, and observed for morphological changes and loss of viability. The 5 logs reduction that occurred in broth at 90 min required 210 min in buffered water. Five characteristic changes observed after spores were exposed 120 min at 90 C in buffered water were: (1) 90% loss of spore viability, (2) < 5% stainability, (3) 76% increase in spore size (as observed by scanning electron microscopy), (4) 21% of spore areas remaining refractile, and (5) an increase of 77% in packed cell volume (PCV). Stainability and PCV changes were recognized only after secondary exposure in broth. Extended heat exposure (3 h at 90 C) resulted in > 99% loss of spore viability and > 99% loss of stainability. After 4 h of heat exposure, 90% of the cells disintegrated. These results suggest that early germinal changes occur concurrently with the early changes in the heat susceptibility of dormant spores.

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The appearance of heat susceptibility is the first in a sequence of events used to measure the degree of spore germination (9,10). The effect(s) of heat are followed by observing the germinal changes occurring in spore suspensions or by enumerating the number of remaining viable organisms.

In some heat studies (4,8,12,13,15), investigators presented wet-heat thermal-death-time curves that were non-linear; other workers (17,19) obtained rate curves that followed first-order kinetics. In our work with Bacillus subtilis var. niger spores, the survival curve at 90 C (Fig. 1) indicates one log loss in viable spore count at the early stage (0 to < 120 min) of heating, but at > 120 min, a drastic reduction in the number of viable organisms is indicated.

Since the onset of heat sensitivity and loss of viability of spores are inseparable events in the study of spore germination, this investigation was initiated to correlate the morphological changes occurring during thermal inactivation of B. subtilis var. niger spores in aqueous suspension.

I. EXPERIMENTAL

Production of spores. B. subtilis var. niger spores were produced and harvested as previously described (1). Harvested spores were stored in 95% ethyl alcohol at 5 C, and under these conditions, no change in viable spore count was noted.

Determination of heat resistance of spores. The heat resistance of spores was determined by the conventional pour plating technique. Spores

were stored in phosphate-buffered water (pH 7.0 and 0.3123 mM), dispersed by sonification, and diluted to the desired concentration. Aliquots of diluted aqueous suspensions (approximately 1.0×10^6 spores/ml) were placed in screw-cap culture tubes (16 x 125 mm), with care being taken not to touch the upper inside tube surfaces with the pipette. Screw-cap tubes were preferred to sealed tubes for convenience and were satisfactory for our purpose since the level of recovery of these experiments (< 100 organisms/ml) did not necessitate using the entire tube's contents.

The tubes were placed in a rack, shaken mechanically in an oil bath, heated for the appropriate length of time and at various temperatures, then cooled for 15 min in a circulating water bath at 4 C. Samples were plated in tryptone glucose beef extract agar and incubated at 35 C for 48 h.

Measurement of packed cell volume (PCV). PCV was measured by the method of Hitchins (11). Dormant and heated (as above) spore suspensions (approximately 1×10^{10} spores/ml) were centrifuged at 1283 x g; the supernatant was discarded, and the pellet reconstituted in 0.1 ml of 1-Octanol (J. T. Baker Chemical Co., 222 Red School Lane, Phillipsburg, N. J. 08865)[1] solution to prevent foaming. Samples were drawn into capillary hematocrit tubes, flame sealed, and centrifuged for 5 min at 24,000 x g. The length of the pellet and the total liquid length in each capillary tube were measured with a Glogau vernier caliper #12 (Glogau and Co., Chicago, Ill.). The PCV was expressed as a percentage by using the formula:

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[1] Mention of commercial products does not imply endorsement by the Food and Drug Administration.

$$PCV = \frac{\text{pellet length (mm)}}{\text{pellet length (mm) + supernatant fluid (mm)}} \times 100$$

Measurement of refractility of spores. The extent of spore darkening was determined by phase contrast microscopy (16,20). A loopful of each heated or nonheated spore sample was spread evenly over a 1-cm diameter circular area on a clean, milk slide (2.5 x 7.6 cm). The film was heat fixed and treated with a 2:1 (v/v) mixture of 95% alcohol and glacial acetic acid for 5 min, after which the mixture was poured off and the film rinsed with water. The prepared smears were mounted in water, sealed under a cover slip with melted Vaspar (10), and examined under a 100 X oil immersion objective (Neofluar phase 100/1.30 Zeiss).

Photomicrographs were taken of the prepared slides using panchromatic film (Kodak plus X, ASA 125). Refractility and nonrefractility of all the spores were measured according to the formula for the area of an ellipse (mm^2):

Total area = π ab; light area = π cd;

Dark area = π ab - π cd.

where, a and c = maximum width

b = maximum length of light ellipse area.

d = maximum length of dark ellipse area.

The percentage of the dark area was calculated as:

$$\frac{(\pi \text{ ab} - \pi \text{ cd}) \times 100}{\pi \text{ ab}}$$

Scanning electron microscopy (SEM) of spores. Spores suspended in aqueous solutions (about 1.0 X 10^9/ml) were heat treated in 16 X 125-mm screw-cap tubes at 90 C for appropriate time intervals. An aliquot of each sample was plated to check for viability, and the remaining portion was placed in a solution of Millonig's phosphate buffer (2X) (Dr. P. S. Lin, personal communication)

and fixed in 2% gluteraldehyde solution. The activity of the organisms was observed using SEM and photomicrographs. To determine changes in spore size, 10 randomly selected spores from each photomicrograph were measured as follows:

$$\text{Volume of a cylinder } (mm^2) = \pi r^2 h,$$

where r is radius and h is height.

Staining of spores. The differential "Cold Method" staining procedure of Bartholomew and Mittwers (3), using malachite green and safranin, was used to find the percentage of germinated and nongerminated spores. Examination was made of each prepared slide under a Zeiss light microscope.

II. RESULTS AND DISCUSSION

The survival curves that result from the inactivation of B. subtilis var. niger spores in aqueous suspensions at various temperatures (Fig. 1 and 2) consist at the early stage of heating of a sector parallel to the abscissa, followed by a rapid decline in the number of viable spores as heat treatment proceeds. The rate of inactivation in broth was more than twice that in buffered water. The 5-log reduction in spore count that occurred after 90 min in broth required 210 min in buffered water.

The mechanism of thermal inactivation of bacterial spores in aqueous suspension is not known; however, one criterion used for measuring spore germination is loss of heat resistance. The rapid reduction in spore viability, succeeding the initial resistance of the spore population to heat (Fig. 1 and 2), could be the result of spore germination.

To determine the sequence of germinal events occurring during the initial heating period, heat-treated as well as nonheat-treated spores were examined microscopically. A definite increase in spore darkening (52%) accompanied by only 15% loss of spore viability was achieved after 15 min at 90 C (Fig. 3). Darkening of spores increased to 84% after 2- and 3-h heat exposure, but total non-refractility was never achieved even when 99% of the spores were no longer viable. The increase in spore darkening during prolonged heat-treatment is dramatically shown in the sequence of phasecontrast micrographs of heat-treated and nonheat-treated spores (Fig. 4a to 4h). Occasionally some heated spores appear to have a more spherical shape than nonheated spores. At 180 min (Fig. 4h) spores appear oblong and spindle-like and some are totally dark.

Swelling of heat-treated spores was demonstrated by PCV measurements. No PCV increase was observed when spores were suspended in buffered water. However, when spores that had been heat-treated in buffered water for 60, 120, and 180 min were exposed briefly (2 min) to tryptose glucose beef extract (TGE) broth, PCV increases of 57, 77, and 62%, respectively, were observed (Fig. 5).

Changes following heating in the size and shape of spores that were observed using SEM are shown in Figs. 6a to 6i. At the lower magnifications (5,000 X), quantitative measurements were made of the spore populations. At higher magnifications (10,000 and 20,000 X) detailed surface features can be seen. At zero time, the general shape of nonheat-treated spores is either spherical or cylindrical (Fig. 6a). Figure 6b (20,000 X) shows some angulated appearance at the end of the spores.

After 1 and 2 h of heat treatment, measurements made from photomicrographs showed a 53% and 75% increase in cell volume. These increases are demonstrated in Fig. 6c and 6d. Notice the significant differences in the diameter of spherical spores (Fig. 6a and 6d).

After 3 h of heat exposure, a decrease in spore volume (- 7.7%) occurred (Fig. 6e). At 4 h of heat exposure, absence of numerous intact spores is illustrated in Fig. 6f and 6g. Numerous smaller cell bodies surrounding a few intact spores are evident.

Another interesting aspect in the SEM study is the effect of a nutrient suspension on spores at 90 C. Notice the "vegetative" cell-like appearance shown in Figs. 6h and 6i (arrows) of some spores. These spores resemble cells in their "outgrowth" stage. Notice the dramatic size differences (cylindrical spores) between spores shown in Fig. 6a and 6h.

After heating, the stainability decreased for spores treated in a manner similar to the PCV studies. Spores lost 95% of their primary stain when exposed briefly to broth following a 2-h heat treatment at 90 C.

The changes in the characteristics that were studied during the course of prolonged exposure of a B. subtilis var. niger spore population to 90 C are compared in Table 1. The viability, refractility, and stainability of spores fell dramatically during the 3 h of heating. Conversely, spore size, whether measured by SEM or PCV, increased as much as 75% during the first 2 h and later decreased as heating was extended.

III. CONCLUSION

This study demonstrates the changes that occur when spores are exposed to a moderately high temperature (90 C) in aqueous suspension and mimic events associated with spore outgrowth and germination. These alterations are induced when dormant spores are exposed at moderate temperatures to solutions that stimulate germination or to sublethal doses of heat for short periods of time.

During the early stage of heating, the stimulatory as well as depressive effects of heat on spore germination are reflected in the minimal loss of viable spores suspended in buffered water at 90 C (33% loss in 1 h). After 2 h there is a rapid decrease of viable spores (93%).

The major event in germination is a change in the coat permeability of dormant spores. Our earlier observations demonstrate that the type of suspending medium and the time-temperature relationships used during heat treatment have a profound effect on spore coat permeability. In the initial phase of heating, the weakening of the spore coat probably takes place slowly, thus providing slower changes in the properties of underlying structures.

Later occurring changes resulting from continued heating (loss of stainability, changes in spore refractility, increases in spore size) correlate with loss of spore viability in the total spore population (Table 1). The results showed a 90% loss of spore viability when these morphological changes mimicked optimum germinal response.

Our findings substantiate work done by others. Uehara and Frank (18) utilized phasecontrast microscopy to show that incompletely germinated semirefractile spores resulted even after an increase in spore

extinction occurred. Hashimoto et al. (10) and Gould (6) each suggested that peripheral changes precede changes deep within the spore during germination. Lefebre and Preiss (5) showed that the region near the surface of the spore is particularly sensitive to low energy electrons and results in spore extinction during germination. However, the area deep within the spore is less sensitive and remained viable. Murrell and Scott (14), and Black and Gerhardt (2) showed that permeability of spores to water progresses parallel to germination.

Loss of the primary stainability (Table 1) may be a chemical reaction associated with the release of some spore constituents during the swelling of the spores, or the weakening of the permeability barriers as heat exposure is extended.

Grecz (7) showed that spore cytoplasmic membranes are damaged after being heat treated, resulting in the destabilization and unmasking of spore-cell structure and cell constituents. This was evident by their puffed, blistered, and cracked appearance seen under electron microscopy. Our use of SEM indicates a similar effect whereby spores swell and then disintegrate during extended heat exposure.

Differences between rates of inactivation of spores, shown earlier, in buffered water and in broth are probably attributed to the presence of a particular nutrient(s) in the broth which initiates germination.

The exact mechanism of thermal inactivation of spores in squeous suspension is complex and diverse; however, our findings are consistent with the increasing evidence of others that inactivation first occurs in the cortex area where the germinal activity also begins.

ACKNOWLEDGMENTS

We thank Dr. Robert M. Twedt for his interest in the research and review of the manuscript. We also thank Mrs. Jean Williams and Mrs. Ruth Dixon for typing the manuscript, and we acknowledge with gratitude the excellent technical assistance of Messrs. Ronald Crawford and Albert Wehby, as well as the excellent editorial assistance of Ms. Loraine S. Good and Mr. J. T. Peeler for his statistical assistance.

LITERATURE CITED

1. Angelotti, R., J. H. Maryanski, T. F. Butler, J. T. Peeler, and J. E. Campbell. 1968. Influence of spore moisture content on the dry-heat resistance of Bacillus subtilis var. niger. Appl. Microbiol. 16:735-745.

2. Black, S. H., and P. Gerhardt. 1962. Permeability of bacterial spores. III. Permeation relative to germination. J. Bacteriol. 83:301-305.

3. Conn., H. J., J. W. Bartholomew, and M. W. Jennison. 1957. The Staining Methods, p. 21-22. In, H. J. Conn (ed.), The Manual of Microbiological Methods by the Society of American Bacteriologists. McGraw-Hill Publ. Co., New York.

4. Fox, K., and B. D. Eder. 1969. Comparison of survivor curves of Bacillus subtilis spores subjected to wet and dry heat. J. Food Sci. 34:518-521.

5. Lefebre, G. M., and J. W. Preiss. 1970. The regional sensitivity of bacterial spores. Radiat. Res. 42:488-497.

6. Gould, G. W. 1970. Mechanism of the inhibition of germination of bacterial spores by γ-irradiation in the presence of iodoacitamide and iodate. J. Gen. Microbiol. 64:301-309.

7. Grecz, N. 1975. Heat resistance of spores related to food. Final report. FDA Grant II, HFF-7.

8. Harnulv, B. G., and B. G. Snygg. 1972. Heat resistance of Bacillus subtilis spores at various water activities. J. Appl. Bacteriol. 35:615-624.

9. Hashimoto, T., W. R. Frieben, and S. F. Conti. 1969. Germination of single bacterial spores. J. Bacteriol. 98:1011-1020.

10. Hashimoto, T., W. R. Frieben, and S. F. Conti. 1972. Kinetics of germination of heat-injured Bacillus cereus spores, p. 409-415. In, H. O. Halvorson, R. Hanson, and L. L. Campbell (eds.), Spores, Vol. 5. American Society for Microbiology, Fontana, Wisconsin.

11. Hitchins, A. D., G. W. Gould, and A. Hurst. 1963. The swelling of bacterial spores during germination and outgrowth. J. Gen. Microbiol. 30:445-458.

12. Hoffman, R. K., V. M. Gambill, and L. M. Buchanan. 1968. Effect of cell moisture on the thermal inactivation rate of bacterial spores. Appl. Microbiol. 16:1240-1244.

13. Licciardello, J. J., and J. T. R. Nickerson. 1963. Some observations on bacterial thermal death time curves. Appl. Microbiol. 11:476-480.

14. Murrell, W. G., and W. J. Scott. 1958. The permeability of bacterial spores to water, p. 26. Seventh International Congress of Microbiologists, Stockholm, Sweden. Abstr.

15. Murrell, W. G., and W. S. Scott. 1966. The heat resistance of bacterial spores at various water activities. J. Gen. Microbiol. 43:411-425.

16. Pulvertaft, R. J. V., and J. A. Haynes. 1951. Adenosine and spore germination: Phase contrast studies. J. Gen. Microbiol. 5:657-663.

17. Rahn, O. 1945. Physical methods of sterilization of microorganisms. Bacteriol. Rev. 9:1-47.

18. Uehara, M., and H. A. Frank. 1967. Sequence of events during germination of putrefactive anaerobe 3679 spores. J. Bacteriol. 94(3):506-511.

19. Watkins, J. H., and C. E. Winslow. 1932. Factors determining the rate of mortality of bacteria exposed to alkalinity and heat. J. Bacteriol. 24:243-265.

20. Wolf, J., and C. M. Thorley. 1957. The effects of various germination agents on the spores of some strains of B. subtilis. J. Appl. Bacteriol. 20(3):384-389.

Table 1. Effect of heat treatment upon the percent distribution of selected characteristics among a population of Bacillus subtilis var. niger spores[a]

Characteristic	Exposure time (h)			
	0	1	2	3
Viability[b]	100.0	66.6	7.0	0.3[c]
Refractility	51.3	10.5	21.0	13.2
Size	100.0	153.6	176.0	92.3
Stainability[d]	100.0	95.0	< 5.0	< 1.0
Packed cell volume	100.0	157.0	177.0	162.0

[a] Suspended in phosphate buffered water at 90 C for various lengths of time.

[b] Measured by plate count method.

[c] 2½ h.

[d] Subcultured in broth for 2 min immediately after heating.

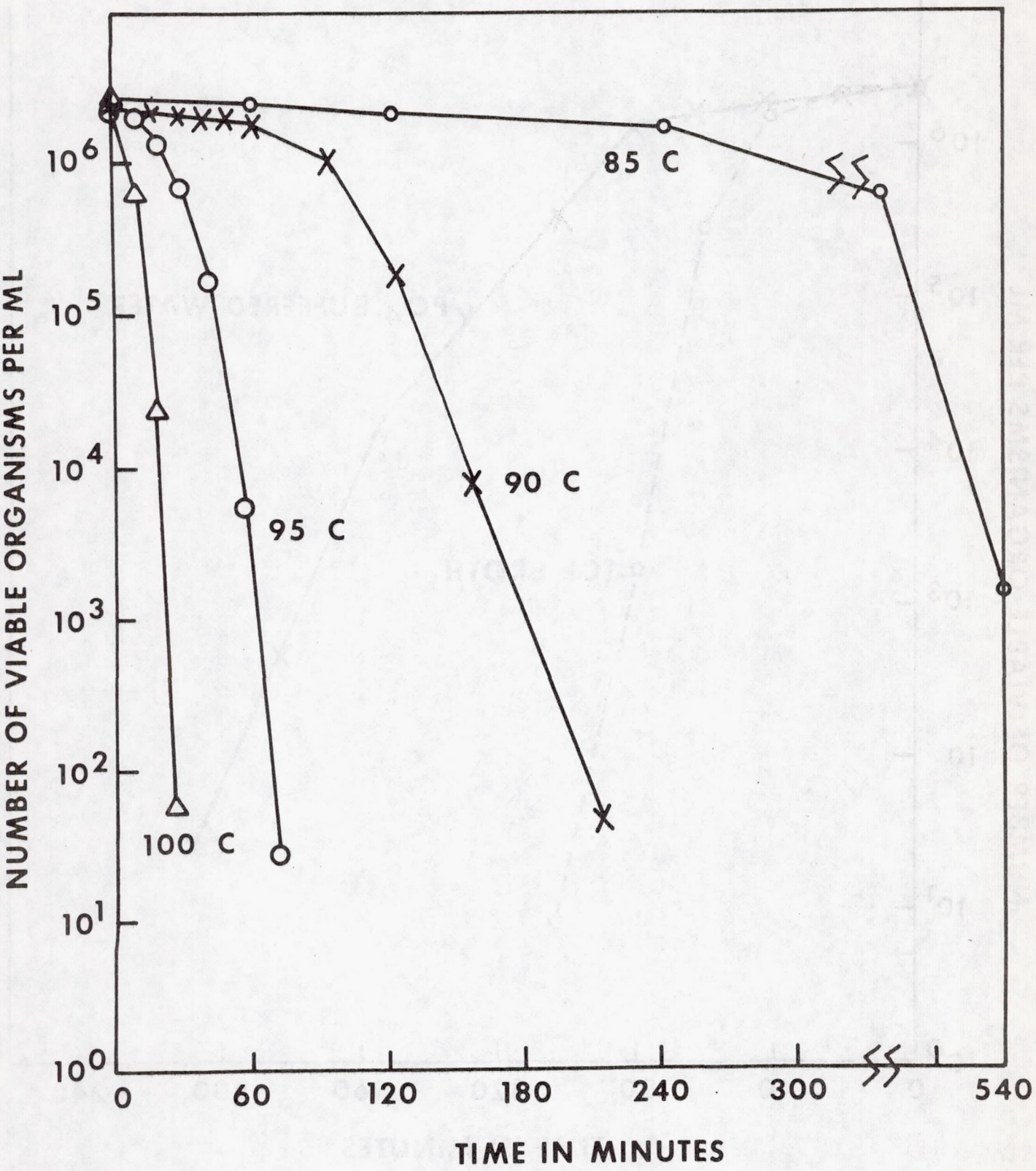


Fig. 1. Heat inactivation of Bacillus subtilis var. niger spores at 85, 90, 95, and 100 C in phosphate-buffered water.

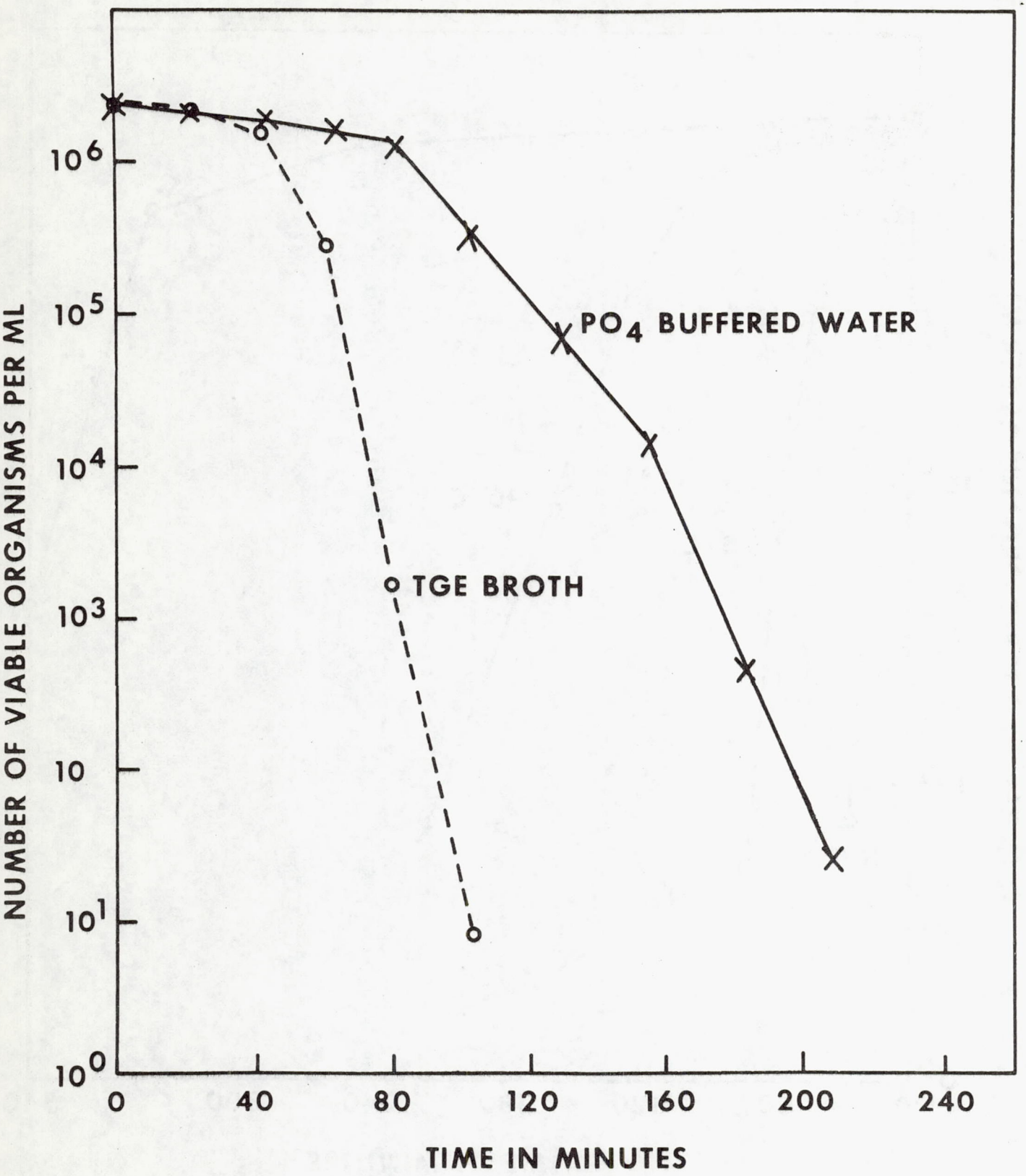


Fig. 2. The effect of suspending medium on the thermal inactivation of Bacillus subtilis var. niger spores at 90 C.

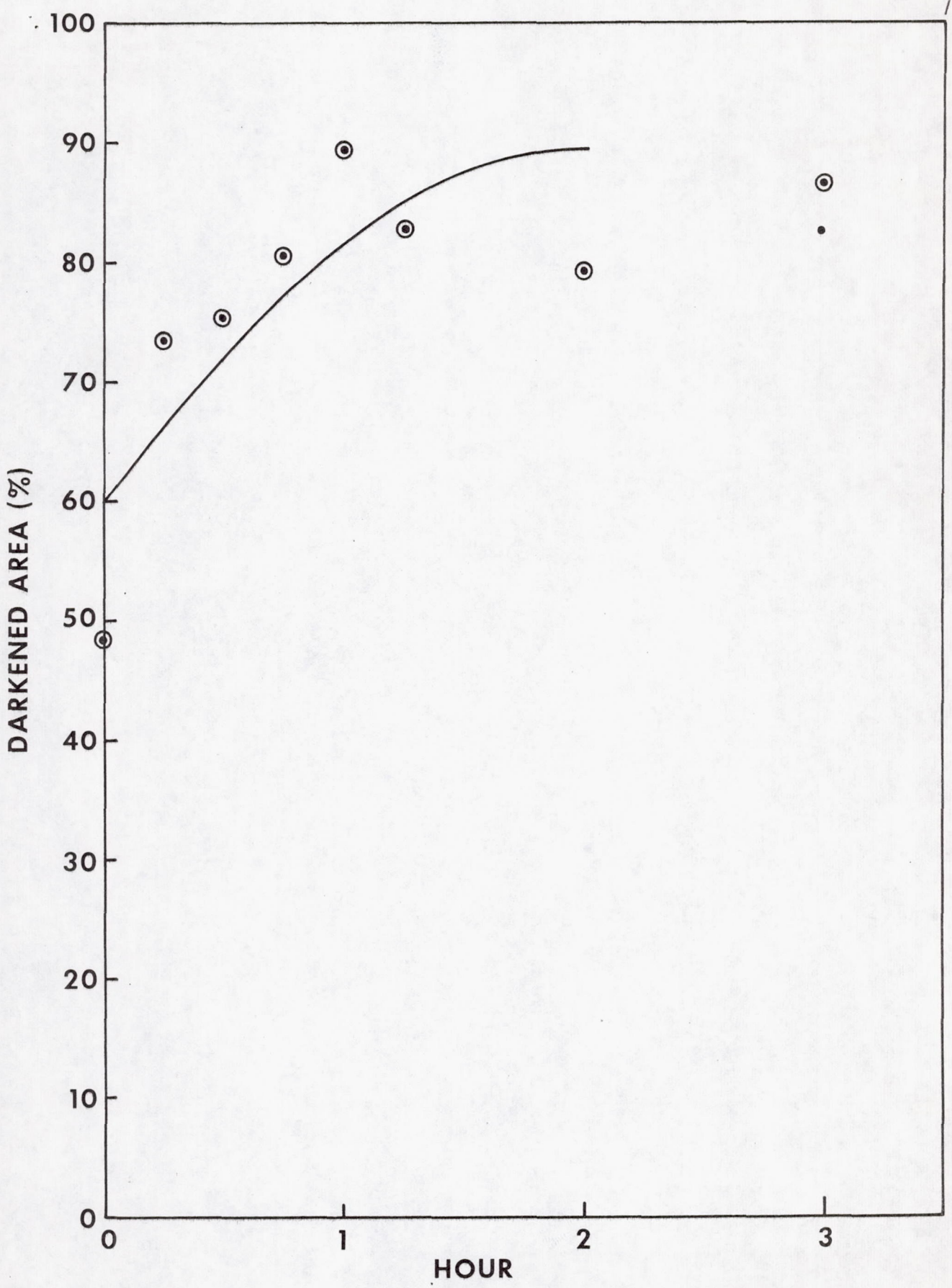
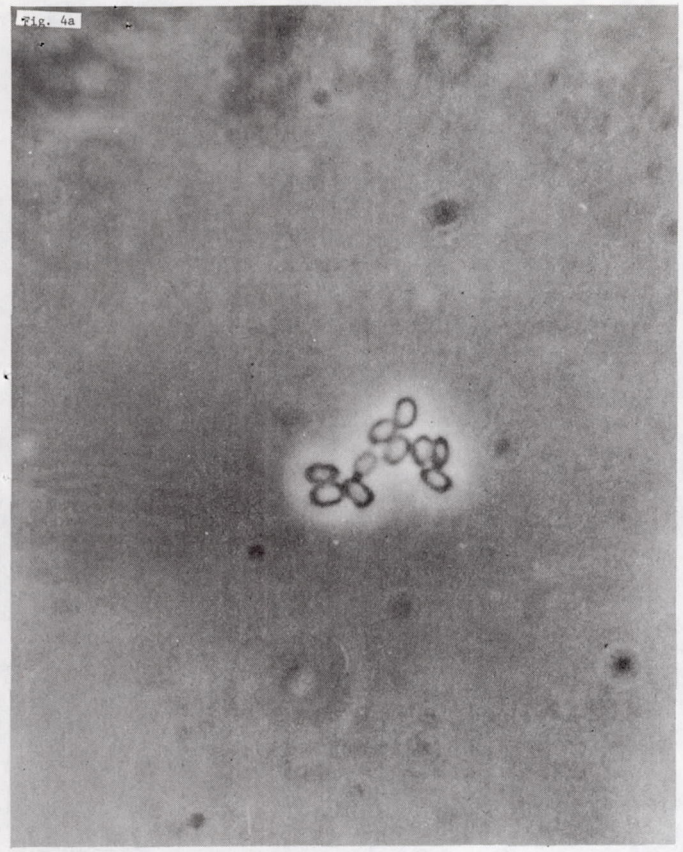
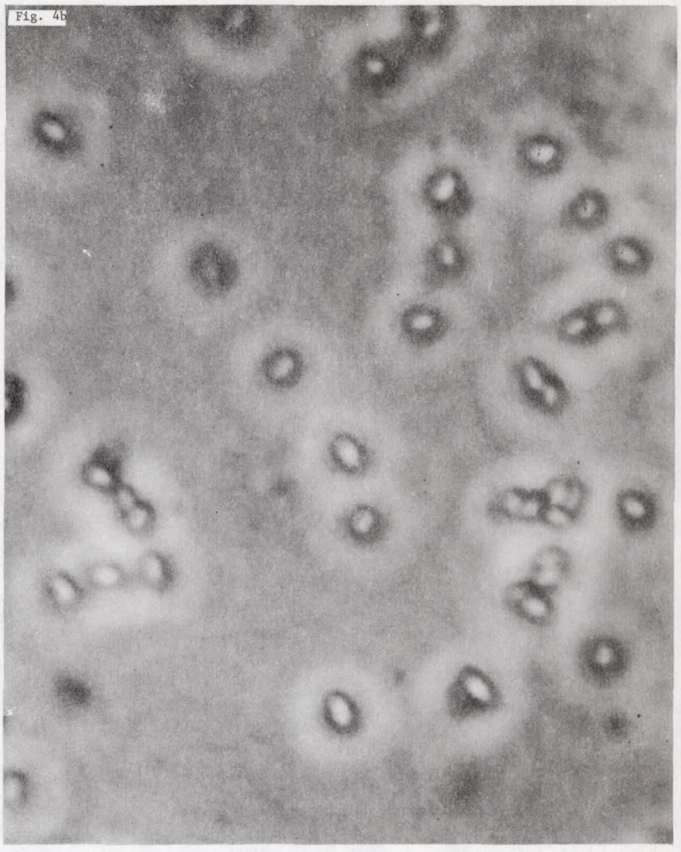
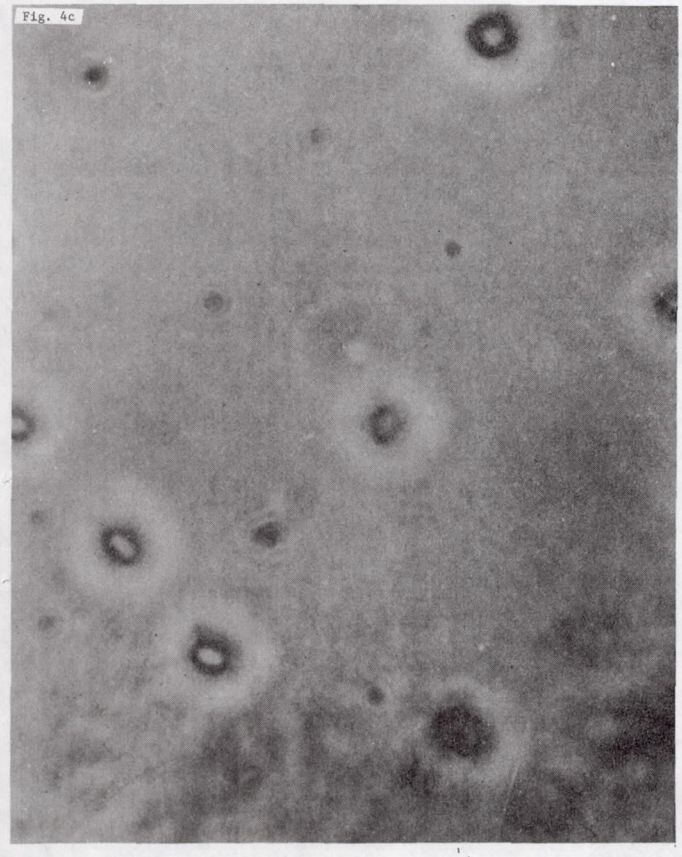


Fig. 3. This is a second polynomial curve fit. The second degree equation has a correlation coefficient of r = + 0.814. This indicates that the percent darkness (◎) is an increasing function of time.







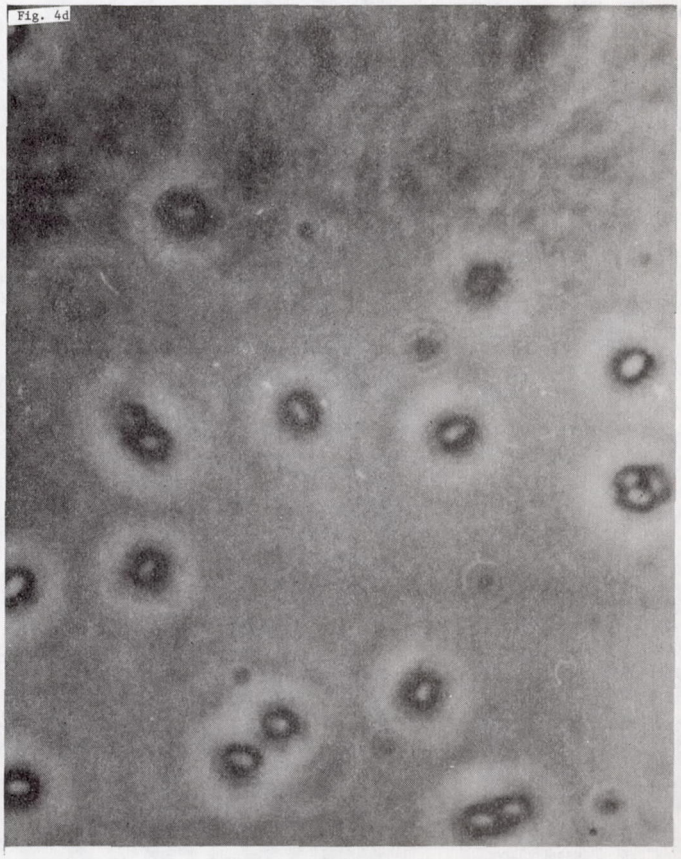


Fig. 4a - 4h. Phase contrast micrographs of Bacillus subtilis var. niger spores. (See text for explanation.)

Fig. 4e

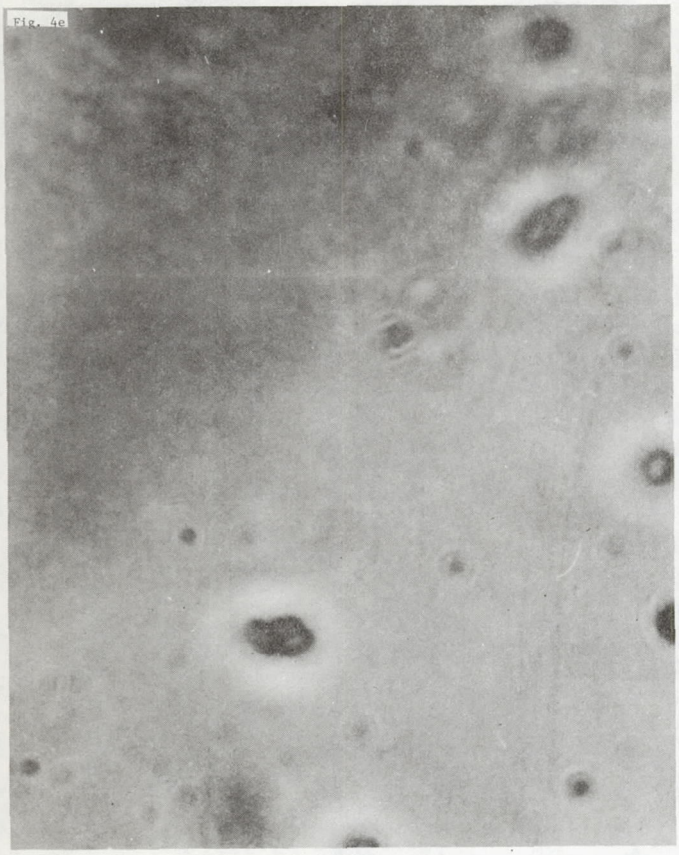


Fig. 4f

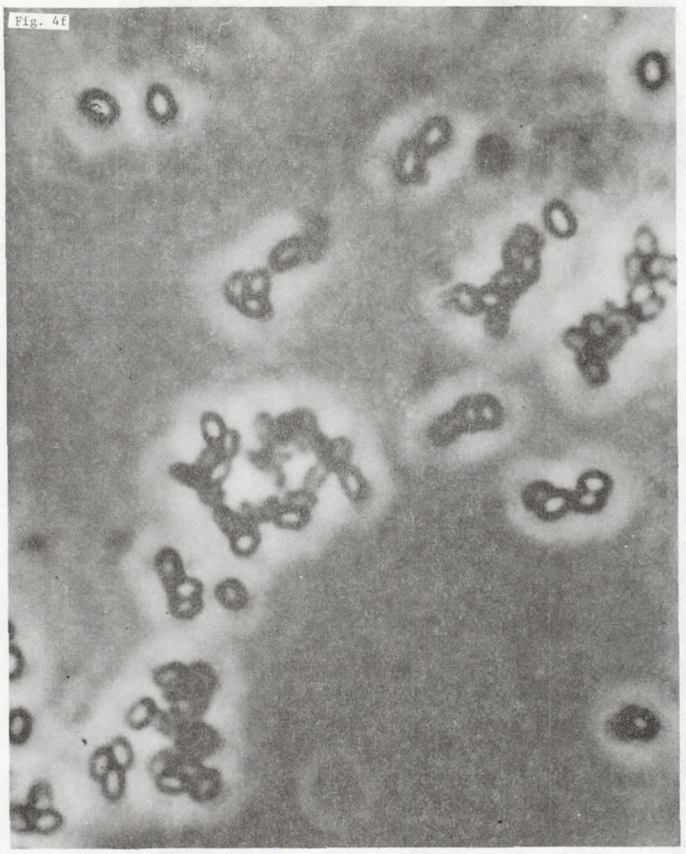


Fig. 4g

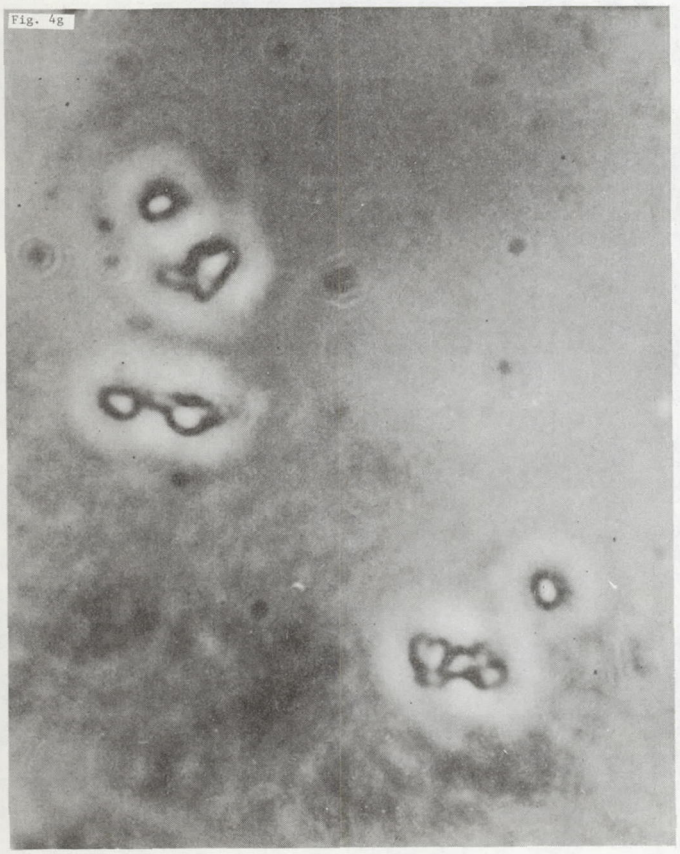
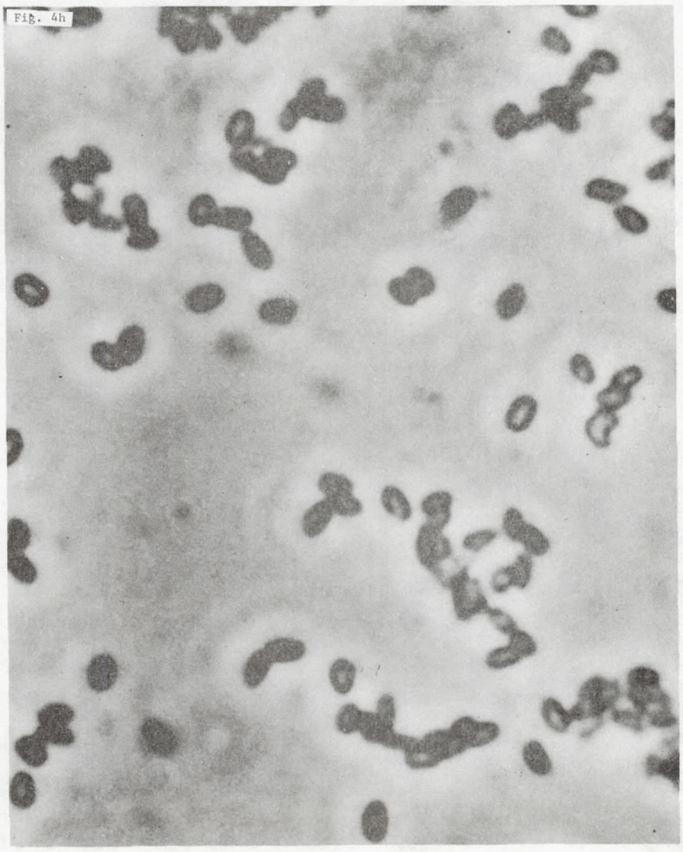


Fig. 4h



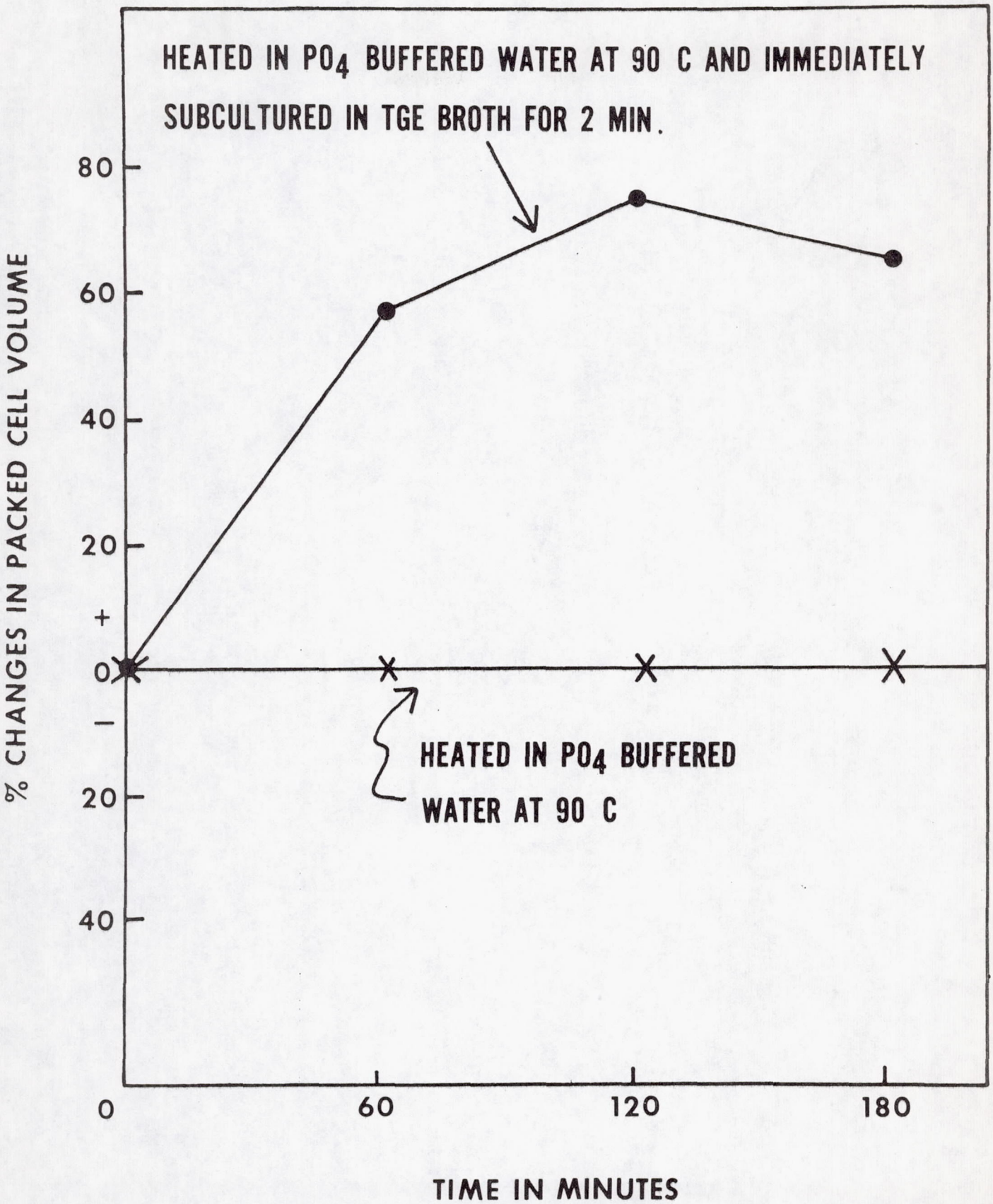


Fig. 5. Effect of suspending medium on the changes in PCV for Bacillus subtilis var. niger spores.

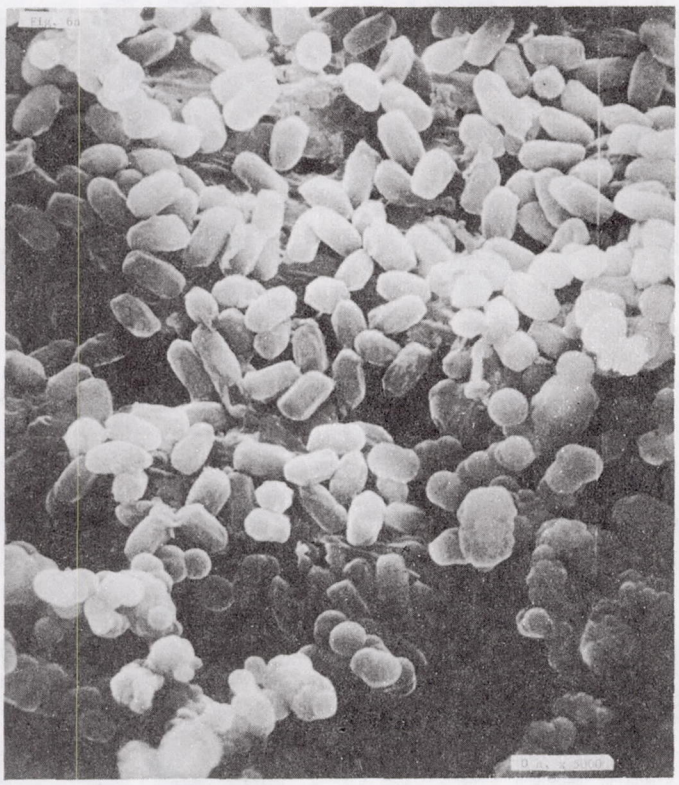


Fig. 6a - 6i. Scanning electron micrographs (x 5000, x 10,000, or x 20,000) of Bacillus subtilis var. niger spores; spores were heat or nonheat treated in phosphate buffered water (Fig. 6a - 6g) or broth (Fig. 6h - 6i) at 90 C.

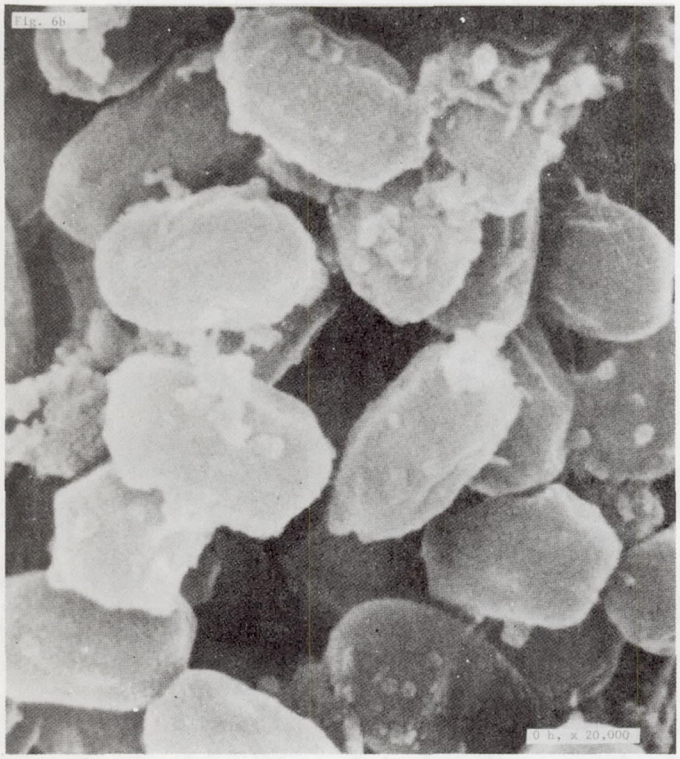


Fig. 6b
0 h, x 20,000

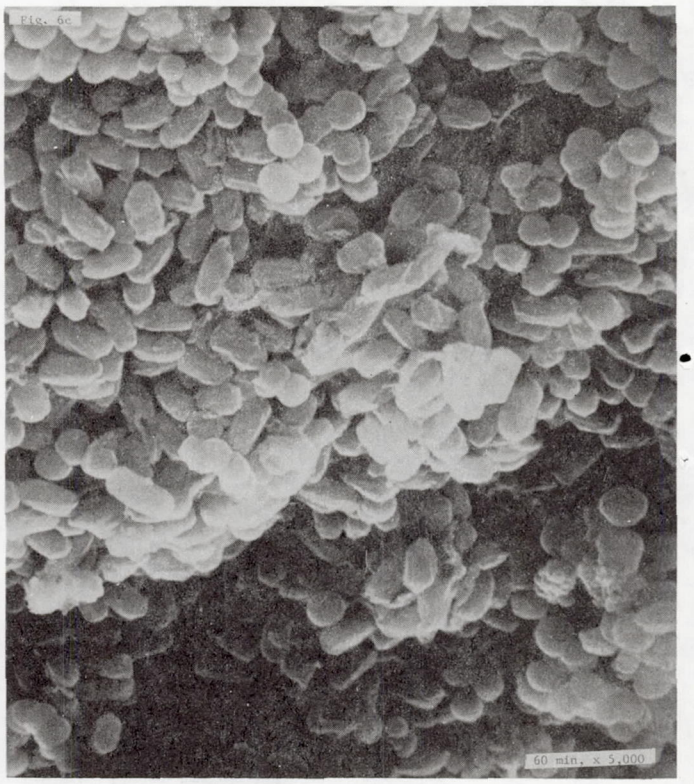


Fig. 6c
60 min, x 5,000

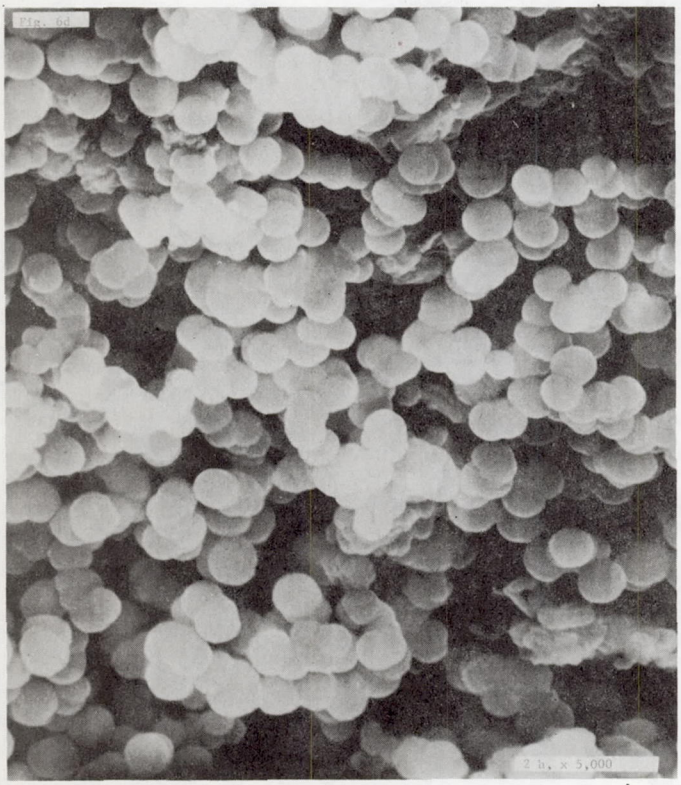


Fig. 6d
2 h, x 5,000

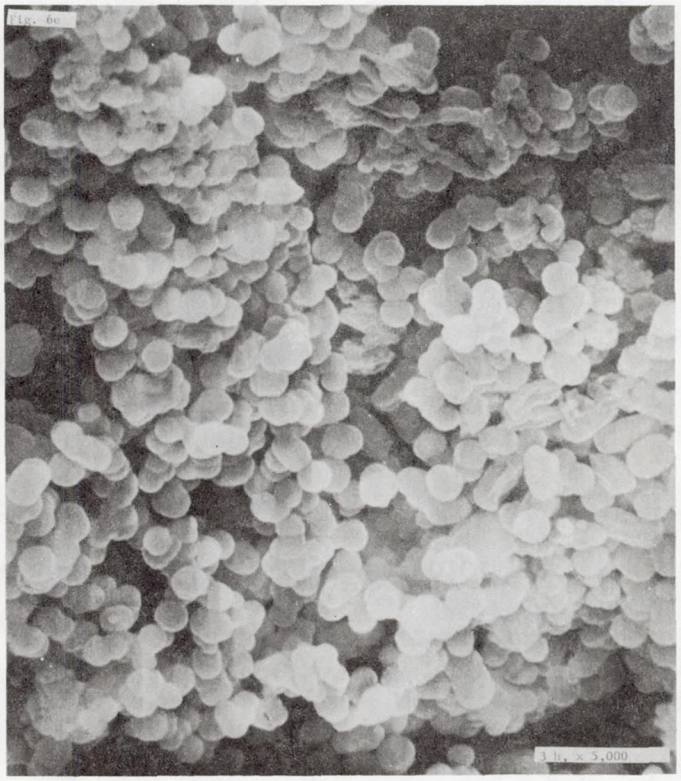
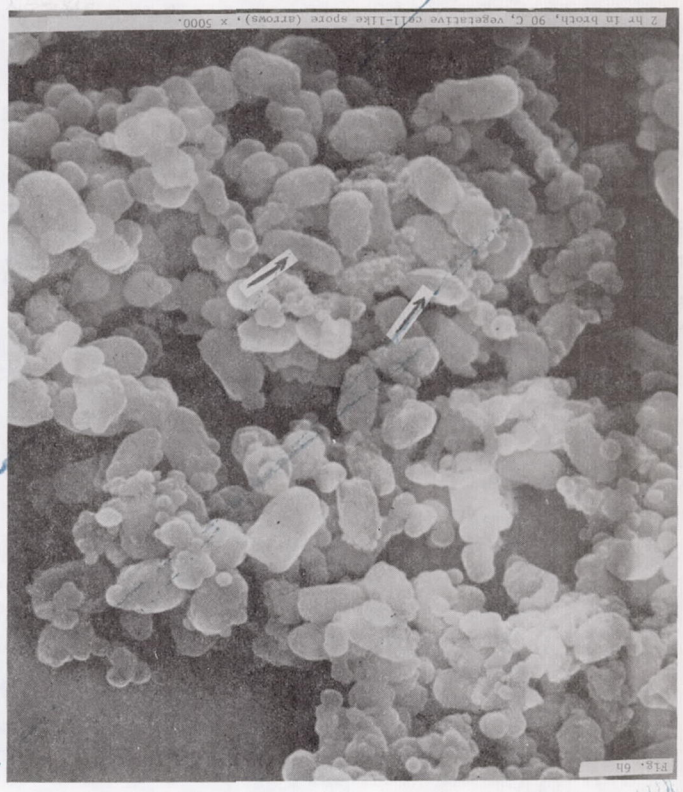
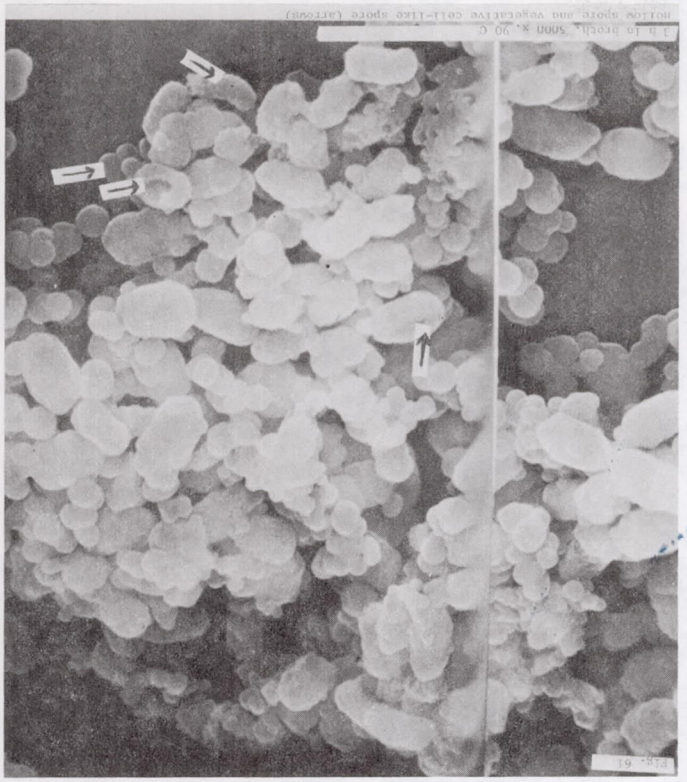


Fig. 6e
3 h, x 5,000



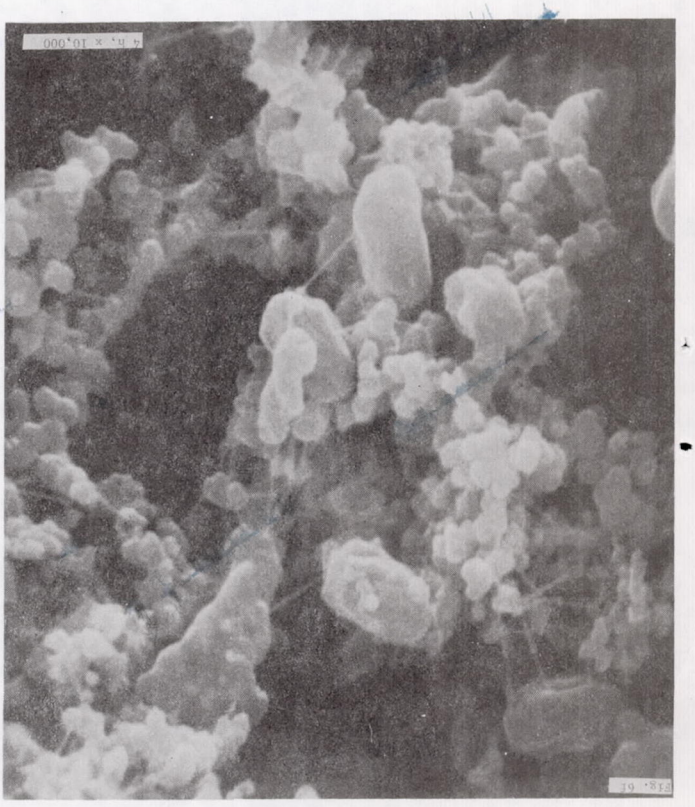
2 hr in broth, 90 C, vegetative cell-like spore (arrows). x 5000.

Fig. 6h



1 h in broth, 5000 x, 90 C
hollow spore and vegetative cell-like spore (arrows)

Fig. 6i



4 h. x 10,000

Fig. 6f

4 h. x 20,000

Fig. 6g